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The Effect of Holothurin on Leucocyte Migration

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Crude Holothurin, purified Holothurin A, and desulfated Holothurin A, water soluble steroid saponins from the Cuvierian organs of the Bahamian sea cucumber *Actinopyga agassizi* Selenka, cause a stimulation of leucocyte migration from buffy layer in capillary tubes in relatively low concentrations and an inhibition of the movements at higher concentrations. The inhibitory properties are compared with similar effects by Ouabain, a non-hemolytic saponin, and by liberated hemoglobin.

The Holothurins had no effect on the migration pattern of the leucocytes in the presence of anaerobic and aerobic inhibitors.

The effect of Holothurin on the amoeboid movements of the leucocytes is discussed in relation to surface alterations and cell membrane permeability.

Introduction

The main purpose of this study was to obtain additional information on the biological properties of Holothurin and its fractions. Many toxic materials obtained from plant and animal tissues cause similar host responses when released from combination with cellular components. The effects of crude Holothurin, Holothurin A, and desulfated Holothurin were studied in systems of mammalian leucocytes present in whole blood or in saline suspensions of white blood cells using the migration technique of Ketchel and Favour (1955). Comparative experiments were made with the saponin Ouabain, while hemolytic properties were investigated in relation to the inhibition of leucocyte activity. By the use of aerobic and anaerobic inhibitors, an estimate was made of biochemical pathways associated with a stimulation of leucocyte migration.

Holothurin is a water soluble, steroid saponin with surface-active properties found in the Cuvierian organ of the Bahamian and West Indian sea cucumber *Actinopyga agassizi* Selenka. The active principle may be obtained from granules in branching filaments of Cuvierian tubules. Crude Holothurin represents dried tubules and analysis shows the presence of glycosides, pigment, cholesterol, insoluble proteins, salts, free amino acids, and polypeptides (Nigrelli and Jakowska, 1960). Holothurin, obtained from the water extract of these granules, consists of steroid aglycones, bound individually to four molecules of monosaccharides (Chanley, *et al.*, 1960). Holothurin A represents 40% of the crude Holothurin and is a cholesterol-precipitated fraction (Nigrelli and Jakowska, 1960) with the empirical formula

$$C_{50\text{-}52}H_{81\text{-}85}O_{25\text{-}26}SNa$$

(Chanley, *et al.*, 1959).

Infrared spectrum analysis indicates a five-or-six-membered ring lactone and one double bond. On acid hydrolysis, Holothurin A yields water-soluble aglycones, sulfuric acid and water soluble reducing sugars (Chanley, *et al.*, 1960). Recent work has shown the presence of a half-esterified sulfate residue ($-OSO_3^-Na^+$), probably attached at some point in the sugar chain. Desulfation of Holothurin A occurs when treated with 0.2M methanolic HCl at 37 C. This results in a neutral product devoid of the sulfate group, while retaining unaltered glycoside-genin bonds (Friess, *et al.*, 1967).

Investigations into properties of Holothurin have shown it to be a powerful neurotoxic agent (Freiss, *et al.*, 1959) and to possess antitumor activity capable of suppressing growth of Sarcoma-180 (Nigrelli, 1952; Nigrelli and Zahl, 1952). Similar effects were noted on Krebs-2 ascites tumors in Swiss mice (Sullivan, *et al.*, 1955). Hemopoietic effects have been demonstrated in *Rana pipiens* (Jakowska, *et al.*, 1958), while hemolytic properties were noted using rabbit red blood cells (Nigrelli and Jakowska, 1960) and human erythrocytes (Thron, 1964).

In the current study, the effect of Holothurin and its fractions in a system of leucocytes displaying amoeboid movement is described. The effect of these compounds on this physiological property was interpreted in relation to surface alterations and cell membrane permeability.

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Materials and Methods

A. *Holothurin solutions*

Stock solutions of crude Holothurin, purified Holothurin A, and desulfated Holothurin A were prepared in physiological saline solution. Final concentrations are listed in Tables 1 through 11. (For Tables, see page 8 *ff.*)

B. *Leucocyte migration*

A modification of the technique developed by Ketchel and Favour (1955) was used throughout this study. Leucocyte ability to migrate by amoeboid motion was measured quantitatively as white blood cells moved away from a leucocyte buffy layer.

The technique utilized a micro-hematocrit formed inside capillary tubes each of which was approximately 7 cm long with an internal diameter of 0.8 ± 0.1 mm. New tubes were used for each experiment.

By means of capillarity, each tube was filled with heparinized blood (0.1 mg heparin/ml blood) in the presence or absence of each test compound, to two-thirds its length. One end of each tube then was heat sealed. Temperature effects associated with this method of sealing did not alter migration patterns. The tube was centrifuged at 3000 rpm for 2 minutes, producing a three component system: a lower layer of packed erythrocytes, an intermediate buffy layer of leucocytes, and an upper layer of plasma (semi-clot). Upon vertical incubation of each tube at 37°C for 5 hours, leucocytes migrated into the plasma layer. Extent of migration from the buffy coat was then measured by means of an ocular micrometer.

Trypan Blue (Tennant, 1964) staining (1% solution) was always performed on leucocytes from the buffy layer after each five hour incubation to estimate the percentage of viable cell populations and data analyzed statistically using Student's test (Snedecor, 1956).

1) *Whole blood containing Holothurin*

Hospitalized psychiatric patients, receiving no medication, served as blood donors. Two ml of heparin (2 mg/ml) in isotonic saline was added to 38 ml venous blood. This final concentration of heparin (0.1 mg/ml blood) delayed clotting. Various amounts of crude Holothurin, Holothurin A, or desulfated Holothurin A in saline or saline alone, were added to the heparinized blood, incubated for two hours at 25°C, and then used to fill each capillary tube.

2) *Leucocyte suspensions containing Holothurin*

Heparinized blood (0.1 mg heparin/ml blood) placed in long stoppered glass tubes, 6x250 mm, were incubated at a 45-degree angle for 45 mins. at 37°C. The plasma-leucocyte layer was then withdrawn, pooled, and gently mixed with a pipette. Varying amounts of crude Holothurin in saline were added to the plasma suspension to attain higher concentrations than possible in whole blood, without hemolysis from erythrocytes. Incubation of the suspension porceeded for 2 hrs. at 37°C in rubber stoppered tubes (15 x 100 mm), after which the suspension was centrifuged at 1500 rpm for 5 mins. and washed once with 5-10 ml heparinized saline. After additional centrifugation at 1500 rpm for 5 mins. the saline solution was discarded and leucocytes resuspended in homologous plasma, using amounts of Holothurin-free heparinized plasma comparable to the original ratio in whole blood.

In order to use the Ketchel and Favour (1955) migration procedure, it was found necessary to have the leucocyte buffy layer overlay packed erythrocytes in each capillary tube. To obtain a leucocyte-plasma-erythrocyte ratio comparable to that which initially existed in the whole blood, heparinized blood was centrifuged in hematocrit tubes at 3000 rpm for 15 mins. From divisions on the tubes and a known total volume, it was possible to estimate the amount of each component initially present in a blood sample.

On this basis, reconstituted leucocyte-plasma-erythrocyte suspensions were placed in capillary tubes and taken through the procedure as described for whole blood. Experience has shown that leucocytes will not migrate when a buffy layer is located at the basal end of each capillary tube. Toxic factors are probably released from the glass tip during the heat sealing process.

3) *The effect of hemolysis*

a) To obtain plasma containing a high concentration of hemoglobin, several ml of heparinized blood (0.1 mg heparin/ml blood) were frozen and thawed three times using dry ice in absolute alcohol. Centrifugation at 3000 rpm for 10 minutes allowed removal of cell debris. The resulting solution consisted mainly of hemoglobin in plasma.

b) Hemolyzed blood prepared with crude Holothurin in a concentration of 20 μg/ml blood, was allowed to remain at 25°C for 1.5 and four hours. The hemolytic action of Holothurin caused a liberation of hemoglobin. After centrifugation at 3000 rpm for 10 minutes, a cell-free solution of hemoglobin remained.

To equate the intensity of liberated hemoglobin in these two plasma samples, optical density readings were made using a Klett-Summerson colorimeter (#54 filter). Heparinized blood in contact with Holothurin for 1.5

hours at 25°C and hemoglobin added to normal plasma to give a corresponding O.D., constituted solutions and readings (A). (See Table 6.) The same procedure was used for (B) solutions, except that Holothurin was allowed to remain in heparinized blood four hours at 25°C. Correspondingly more hemoglobin was then required in plasma to give higher O.D. readings in (B).

After establishing the exact amount of hemoglobin (liberated by freezing and thawing or Holothurin treatment) necessary to give comparable concentrations in whole blood, capillary tubes were filled and incubated at 37°C for five hours. Leucocyte migration was then measured by means of an ocular micrometer.

4) *Crude Holothurin versus Ouabain*

Experiments were designed to show the effect of a non-hemolytic saponin (Ouabain) in relation to a hemolytic saponin (Holothurin) on leucocyte migration.

To obtain the same molar concentration, calculations based on molecular weights of Ouabain and Holothurin were made to estimate the amount of each compound necessary to give a final concentration of 1.4×10^{-5}M in heparinized blood. Stock solutions of each compound were prepared in saline and appropriate dilutions added to heparinized blood. Capillary tubes were then filled, sealed, centrifuged, and incubated for five hours at 37°C.

5) *The influence of enzymatic inhibitors*

Freshly prepared saline solutions of the following inhibitors were added to heparinized blood and allowed to remain at 25°C for two hours: Sodium fluoride, iodoacetic acid (sodium salt), 2,4-dinitrophenol, and potassium cyanide. Concentrations are given in Tables 8, 9, 10, 11. Crude Holothurin, at a final concentration of 1 μg/ml blood was added in some cases. All quantities of inhibitors and Holothurin in blood were of volumes less than one part inhibitor or Holothurin to four parts of heparinized blood. Saline controls always contained the same volume ratio. After filling, sealing, and centrifuging (3000 rpm for two minutes) each capillary tube was incubated in a vertical position for five hours at 37°C.

Results

In all cases with crude Holothurin, Holothurin A or desulfated Holothurin A, low concentrations showed a significant increase in the rate of leucocyte amoeboid motion. The stimulation by crude Holothurin (Table 1) occurred mainly in the range of 0.1 to 4 μg/ml blood. Inhibition by crude Holothurin (Table 2) resulted in a decrease in leucocyte migration, principally at levels between 16 and 22 μg crude Holothurin/ml blood. Trypan Blue staining showed the presence of viable cells in all experiments. Effects were not due to massive alterations in the test system; however, considerable hemolysis was noted in tubes treated with higher concentrations of Holothurin. No significant results were observed in concentrations ranging from 1 to 14 μg/ml.

Holothurin A was found to be effective in lesser amounts, producing a stimulation at a concentration of 0.02 μg/ml and a decrease in the rate of amoeboid motion at a concentration of 0.1μg/ml. Movement of leucocytes was completely inhibited at a concentration of 20 μg/ml, with the subsequent liberation of inhibitory concentrations of hemoglobin from erythrocytes.

When desulfated Holothurin A is compared to crude Holothurin and Holothurin A it will be noted that the biological activity of this compound has been greatly changed. Thus, a stimulation in migration will occur at 1μg/ml, while concentrations greater than 100 μg/ml were necessary to inhibit leucocyte motion.

Observations indicate that the hemolytic properties of crude Holothurin in higher concentrations may have some effect on leucocytes in whole blood (Table 5). A crude Holothurin concentration of 40 μg/ml leucocyte suspension (23 μg/ml blood) does not significantly alter the rate of white blood cell migration, however concentrations from 100-1000 μg/ml leucocyte suspension (greater than 58 μg/ml blood) will significantly decrease the rate of motion. A significant alteration in cell viability occurred in the presence of higher concentrations of Holothurin when levels greater than 100 μg/ml leucocyte suspension were reached.

To substantiate the fact that crude Holothurin *per se* had affected leucocyte mobility, experiments were designed to compare results obtained when the non-hemolytic saponin Ouabain was used in the same concentration as the hemolytic saponin (crude Holothurin). Results showed no significant differences between the two saponins at a concentration of 1.4×10^{-5}M (16 μg/ml blood). (Table 8.) The data indicate approximately the same decrease in migration when compared with the control. Trypan Blue staining demonstrated the viable nature of these cells within an acceptable range. No gross mortality had caused alterations in the rate of leucocyte migration.

Holothurin is known to possess strong hemolytic properties with concentrations as linear functions of the red blood cell count and reaction time (Thron, 1964). The effect of homologous hemoglobin, in the absence of crude Holo-

thurin, was found to affect leucocyte migration only in concentrations (O.D.) comparable to that produced by crude Holothurin (O.D. 250) in contact with red blood cells for four hours at 25°C. The hemoglobin concentration (O.D. 103) comparable to crude Holothurin hemolysis after contact for 1.5 hours did not affect leucocyte migration. It appears that the amount of hemoglobin liberated by crude Holothurin after four hours will significantly decrease the rate of leucocyte motion (Table 6).

The effect of selected inhibitors on white cell migration was studied with the hope of obtaining information on metabolic pathways in the presence and absence of crude Holothurin. Both anaerobic inhibitors (sodium fluoride and iodoacetate) depressed migration in concentrations less than 1×10^{-3}M, while the aerobic inhibitors (2,4-dinitrophenol and potassium cyanide) required greater amounts (1×10^{-2}M or more) to produce a decrease. In the case of dinitrophenol, solubility problems prevented an accurate estimation of the amount required to produce an inhibition.

A study of inhibitors, in the presence of crude Holothurin, was undertaken in an attempt to explain the stimulation of migration resulting from use of low concentrations (8.8×10^{-7}M or 1 μg/ml blood) of this compound. In all cases with both aerobic and anaerobic inhibitors, a decrease in migration occurred in the presence of crude Holothurin. When compared to the rate of leucocyte mobility in the absence of inhibitors, it is possible to state that crude Holothurin stimulation is the result of both aerobic and anaerobic metabolism. Leucocyte nonviability was not a factor since Trypan Blue staining indicated a cell survival greater than 95%. All concentrations used allowed each experiment to be carried out under optimal conditions.

Discussion

The biological and chemical activities of Holothurin put this compound in the class of steroid saponins (Nigrelli and Jakowska, 1960) with surface-active properties (Seeman, 1967; Thron, 1964) and ability to alter cell membrane structure. High surface activity of echinoderm toxins, structural arrangement, and chemical properties contribute to their ability to penetrate membranes and demonstrate an affinity for cellular components (Ruggieri, 1965). These properties are significant in the study of Holothurin action on leucocytes in relation to systems incorporating surface phenomena, such as amoeboid movement.

By definition (White, *et al.*, 1964), all saponins lower surface tensions and contribute to cytolytic effects when used in sufficient quantity. The work of Ponder and McLeod (1936) has shown that saponin hemolytic substances will affect white cells in a manner similar to their effect on red cells. Current experimental data using suspensions of leucocytes confirms these earlier reports. Crude Holothurin in concentrations greater than 100 μg/ml will cause a certain percentage of white cell disintegration, decreased cell counts, and altered cell viability.

Since Holothurin is chemically a member of the saponin family, it is expected that this compound will show activity towards cell membranes. Studies by Friess, *et al.*, (1960) postulate that the Holothurin effect may be a nonspecific surface action on cell membranes or that it could be a specific attack at one or more sites in the membrane. The demonstration that Holothurin can produce cytotoxic effects on leucocytes, as shown by Trypan Blue uptake and altered morphology in higher concentrations, would suggest that Holothurin *in vitro*, and possibility *in vivo*, can affect integrity and permeability of this particular cell type. The migration system had advantages over others since it utilized a buffy layer of leucocytes from which isolated cells could move away from the main population.

It has been suggested (Parpart and Ballentine, 1952) that cell membrane structure is a mosaic of cylinders containing phospholipid and cholesterol surrounded by a protein meshwork. Observations by Dourmashkin, *et al.*, (1962) have shown that, in the presence of suitable agents, extensive re-arrangement of these membrane lipids may occur. The original membrane is rendered more permeable by incorporation of saponin, thereby forming a more permeable structure with spaces 90 Angstroms across that appear to contain water. Saponin hemolytic activity is thought to depend on these spaces in the lipid component of cell membranes. Alterations in lipid structures of this kind are important in controlling the permeability of cells under physiological conditions. It is therefore suggested, that changes in leucocyte permeability in the presence of Holothurin could be responsible for several effects observed during the current study with this compound. Holothurin is known to be a surface active agent and it is suspected that a change in cell permeability and eventual destruction of Holothurin-treated leucocytes (in concentrations greater than 100 μg/ml) are initiated by reactions on the cell surface. In addition, Holothurin may alter cell metabolism by virtue of its action at cell surfaces to allow freer passage of this compound to an intracellular environment.

Treatment of human leucocytes with crude Holothurin, Holothurin A, or desulfated Holothurin A resulted in a stimulation of migration as well as inhibition of phagocytic motility. Concentrations were chosen which could affect these physiological properties but not cause cell death in most cases. Results may be explained on the basis of structure, surface properties, or by consideration of the role of chemical interaction between a surface-active agent (Holothurin) and the cells involved. Chemical approaches to amoeboid movement have been lacking; however, it is suspected that Holothurin may enhance migration through a stimulation of leucocyte glycolysis. It is well established that certain substances, as bacterial endotoxins, hormones, and polysaccharides (Woods *et al.*, 1961), produce a glycolytic stimulation and that this stimulation can be associated with an increase in functional capacities of the cells.

On the basis of chemical structure, it will be noted that the concentration of desulfated Holothurin A required to produce a decrease in migration was considerably higher (greater than 100 μg/ml) than that necessary to inhibit leucocyte mobility in the presence of crude Holothurin or Holothurin A. Certain structural configurations of Holothurin modify leucocyte motility in systems involving physiological response. The action of a saponin devoid of the acidic sulfuric acid group may be interpreted in view of its ability to combine with basic groups of membrane proteins. It has been noted by Friess, *et al.*, (1967) that the anionic nature of Holothurin facilitates permeability through membranes, allowing greater speed of action and therefore a more complete reaction in a given time. Desulfated Holothurin A demonstrates an impeded degree of membrane permeation due to a separation of lipid and polysaccharide tissue phases. Toxic manifestations accordingly would be inhibited during the initial phases of interaction within cells. Friess, *et al.*, (1967), also note that a resulfation of desulfated Holothurin may be necessary for this compound to become biologically active.

An alternative approach to explain the stimulation of leucocyte migration resides in an area related to the lytic properties of Holothurin. Holothurin has a strong hemolytic potency since it has a high affinity for erythrocytes. In suspensions of heparinized blood, where both erythrocytes and leucocytes were present, low concentrations of Holothurin were probably taken up by red cells, leaving little or no free lysin to act on the white cells. More Holothurin caused greater absorption by red cells at a constant number; however more free lysin also remained in solution to act on the leucocytes. Under these conditions a stimulation of leucocyte migration is merely the rate occurring where extremely small amounts of Holothurin have remained free in the system (less than 0.02-4 μg/ml). Eventually a concentration of free Holothurin is reached that will be too great to cause a stimulation but not sufficient to produce an inhibition. This will occur in a suspension of blood initially receiving 6-14 μg of crude Holothurin/ml blood, and is referred to as the intermediate range. In crude Holothurin concentrations initially greater than 16 μg/ml blood, much free saponin will be available to inhibit leucocyte activity. As the concentration of this compound is further increased, results (Table 5) indicate much cell destruction concomitant with a significant decrease in leucocyte mobility and the presence of numerous non-viable cells. On microscopic examination, distortion of cell morphology, altered staining properties and fragmentation were evident. Macroscopically at a concentration of 580 μg crude Holothurin/ml blood, viscous turbid solutions of leucocyte debris resulted after approximately one hour in the presence of this compound. It is obvious that cell integrity was being altered, membranes were being attacked, and leucocyte structure was being destroyed.

The effect of liberated hemoglobin also was studied in the migration system. Recent studies by Rideal & Taylor (1958) support the view that hemolysis, caused by saponins, involves adsorption of hemolytic agents on the cell wall. This adsorption alters bound cholesterol, with eventual cell wall destruction and release of hemoglobin. Rate of liberation is dependent on the concentration of saponin present in the system, and a period of time is required for all hemoglobin to be released from the erythrocytes. Table 6 shows the effect of hemoglobin on leucocyte migration. Low concentrations of hemoglobin do not alter migration patterns whereas concentrations attained after four hours, using a crude Holothurin concentration of 20 μg/ml, seem to display an inhibitory effect. Additional studies with leucocyte suspensions (Table 5) indicate that free hemoglobin has been a factor to depress migration in crude Holothurin concentrations of approximately 20 μg/ml blood (Table 2). To further substantiate this conclusion, the non-hemolytic saponin Ouabain was used in a system of heparinized blood (Table 7) at a lesser concentration (1.4×10^{-5}M). Migration results at this level of saponin are due to an effect of the compound and not to the presence of hemoglobin, assuming its action on cell membranes and biochemical processes is similar to that of crude Holothurin. No significant differences were noted between crude Holothurin

and Ouabain when used in the same concentration (Table 7). Both saponins were able to depress significantly leucocyte migration when compared to saline controls.

Several inhibitors were studied in the migration system to further elucidate factors involved in leucocyte metabolism in the presence of Holothurin. A decrease in migration may be explained as the result of inhibition of either glycolytic or oxidative respiratory metabolism of leucocytes. In present studies two anaerobic inhibitors, iodoacetate (8×10^{-5}M) and sodium fluoride (1×10^{-3}M), were found to be most effective towards inhibiting leucocyte mobility. The aerobic inhibitors, 2,4-dinitrophenol and potassium cyanide, were not inhibitory until concentrations greater than 1×10^{-2}M were reached.

The fact that leucocyte metabolism is mainly glycolytic and mobility is inhibited by glycolytic inhibitors would imply that the energy provided by glycolysis is a factor in amoeboid movement. The use of greater amounts of aerobic inhibitors, noted in current studies, would further suggest that aerobic systems affected by cyanide and dinitrophenol also contribute to the production of energy.

Crude Holothurin in a concentration of 1 μg/ml used concomitantly with these inhibitors showed the same migration patterns as inhibitors in the absence of Holothurin. However, a stimulation resulting from a low concentration of crude Holothurin, was evident in all experiments where this saponin was used in the absence of inhibiting compounds. The stimulation of leucocyte migration in the presence of crude Holothurin at low concentrations (1 μg/ml) was due to both aerobic and anaerobic metabolism.

Summary

1) A stimulation of leucocyte migration occurred in the concentration range 0.1-6 μg crude Holothurin/ml blood, at 0.02 μg Holothurin A/ml blood and 1.0 μg desulfated Holothurin A/ml blood.

2) An inhibition of leucocyte migration was produced in concentrations of 16-58 μg crude Holothurin/ml blood, at 0.1 μg Holothurin A/ml blood and 300 μg desulfated Holothurin A/ml blood.

3) Ouabain, a non-hemolytic saponin, inhibited leucocyte migration to the same extent as crude Holothurin when used in a concentration of 1.4×10^{-5}M. (16 μg/ml).

4) Liberated hemoglobin was found to be inhibitory in high concentrations, while lesser amounts did not alter white cell mobility.

5) Crude Holothurin (1.0 μg/ml blood) used concomitantly with anaerobic inhibitors NaF and iodoacetic acid and with aerobic inhibitors 2,4-dinitrophenol and KCN, showed no alteration in migration patterns different from that obtained in the absence of this saponin. Both aerobic and anaerobic metabolism are important for the stimulation of leucocyte migration in the presence of low concentrations of crude Holothurin.

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Table 1. Crude Holothurin Stimulation of Leucocyte Migration Using Whole Blood

Concentration of Holothurin in μg/ml	Holothurin + leucocytes (migration in mm)	Saline* + leucocytes (migration in mm)	Per cent viable Holothurin-treated leucocytes	N	P
0.1	1.94	1.63	100	30	< .001
	2.55	2.38	–	30	< .001
	2.49	2.11	–	30	< .01
	1.88	1.79	–	30	< .1
0.5	2.68	2.11	–	30	< .001
	2.02	1.63	100	30	< .001
	2.22	2.10	–	30	< .001
	2.61	2.38	–	30	< .001
	1.96	1.79	–	30	< .01
1.0	1.97	1.63	99-100	30	< .001
	2.01	1.79	–	30	< .001
	1.50	1.34	–	30	< .001
	2.78	2.11	–	30	< .001
	1.98	1.79	–	30	< .001
	1.89	1.84	–	30	< .3
2.0	2.09	1.63	–	30	< .001
	1.47	1.34	–	30	< .001
	1.81	1.79	–	30	< .8
	1.82	1.79	–	30	< .6
3.0	2.18	1.63	–	30	< .001
4.0	1.90	1.79	99-100	30	< .02
	2.16	2.02	–	30	< .1
6.0	1.84	1.79	–	30	< .5

* Control cells always showed 95-100% viability.

Table 2. Crude Holothurin Inhibition of Leucocyte Migration Using Whole Blood

Concentration of Holothurin in μg/ml	Holothurin + leucocytes (migration in mm)	Saline* + leucocytes (migration in mm)	Per cent viable Holothurin-treated leucocytes	N	P
0.1	1.72	1.84	–	30	< .01
0.5	1.79	1.84	–	30	< .2
1.0	2.34	2.38	–	30	< .3
	1.77	1.79	99-100	30	< .8
2.0	2.09	2.38	–	30	< .001
4.0	1.73	1.76	–	30	< .7
	1.74	1.79	99-100	30	< .6
6.0	2.08	2.08	100	30	< .1
	1.20	1.30	–	30	< .2
8.0	1.19	1.30	97	30	< .4
	1.77	1.79	–	30	< .9
10.0	1.18	1.30	97-100	30	< .3
12.0	1.19	1.30	90	30	< .2
14.0	1.18	1.30	95	30	< .2
16.0	1.52	2.12	90	30	< .001
	1.06	1.30	90-95	30	< .02
18.0	0.66	1.30	90	30	< .001
20.0	0.70	1.30	90-95	30	< .001
22.0	0.51	1.30	90	30	< .001

* Control cells always showed 95-100% viability.

Table 3. The Effect of Holothurin A on Leucocyte Migration Using Whole Blood

Concentration of Holothurin A in μg/ml	*Holothurin A + leucocytes (migration in mm)*	*Saline* + leucocytes (migration in mm)*	*Per cent viable leucocytes*	*N*	*P*
20**	none	1.62	98-100	28	—
0.1	1.53	1.80	99-100	20	< .001
0.025	1.46	1.51	99-100	23	< .05

* Control cells showed 100% viability.
** Much hemolysis present.

Table 4. The Effect of Desulfated Holothurin A on Leucocyte Migration Using Whole Blood

Concentration of desulfated Holothurin A in μg/ml	*Desulfated Holothurin A + leucocytes (migration in mm)*	*Saline* + leucocytes (migration in mm)*	*Per cent viable leucocytes*	*N*	*P*
1	1.24	1.70	100	33	< .01
20	1.69	1.62	100	28	< .8
100	1.38	1.43	100	34	< .4
300	0.68**	1.42	70	7	< .001

* Control cells showed 100% viability.
** Slight amount of hemolysis.

Table 5. Crude Holothurin Inhibition of Leucocyte Migration Using White Blood Cell Suspensions

Holothurin concentration in WBC suspension, μg/ml leucocytes	*Comparable Holothurin concentration in whole blood, μg/ml whole blood*	*Holothurin-treated leucocytes (migration in mm)*	*Saline-treated leucocytes* (migration in mm)*	*Per cent viable Holothurin-treated leucocytes*	*N*	*P*
40	23	1.54	1.67	99-100	15	< .2
100	58	0.90	1.67	85	20	< .001
300	174	0.66	1.67	30-50	14	< .001
1000	580	0.49**	1.67	5***	—	< .001

* Control cells showed 99-100% viability.
** Few cells.
*** Most cells disintegrated.

Table 6. The Effect of Hemoglobin on Leucocyte Migration

Compound	*Lapsed time of contact*	*Klett O.D.*	*Average migration (in mm)*	*Per cent viable*	*N*	*P*
Crude Holothurin (20 μg/ml)	1.5 hrs.	107	1.50	100	29	
Hemoglobin (A)	—	103	2.44	100	20	< .8
Saline	—	—	2.42	100	34	
Crude Holothurin (20 μg/ml)	4.0 hrs.	250	1.37	99	25	
Hemoglobin (B)	—	270	1.97	100	20	< .001
Saline	—	—	2.42	100	34	

Table 7. Leucocyte Migration in the Presence of Crude Holothurin and Ouabain

Compound	*Concentration*	*Average migration (in mm)*	*Per cent viable*	*N*	*P*
Crude Holothurin	1.4×10^{-5}M	1.52	90	20	< .5
Ouabain	1.4×10^{-5}M	1.44	92-95	31	
Saline	—	2.12	95-100	28	

Table 8. Leucocyte Migration in the Presence of Crude Holothurin and Sodium Fluoride

Compound	*Concentration*	*Average migration (in mm)*	*Per cent viable*	*N*
1) Holothurin (1 μg/ml)	8.8×10^{-7}M	1.86	99-100	25
2) Sodium fluoride	8.0×10^{-5}M	1.65	98-99	31
3) Holothurin (8.8×10^{-7}M) + NaF	8.0×10^{-5}M	1.68	100	29
4) Holothurin (8.8×10^{-7}M) + NaF	1.0×10^{-4}M	1.61	99	30
5) Holothurin (8.8×10^{-7}M) + NaF	1.0×10^{-3}M	1.03	98-99	28
6) Saline	—	1.63	98	28
7) Sodium fluoride	8.8×10^{-7}M	1.72	99	25
8) Sodium fluoride	1.0×10^{-3}M	1.10	99-100	28
9) Saline	—	1.73	99-100	28

1) versus 6); $P < .001$
2) versus 6); $P < .6$
3) versus 6); $P < .3$
4) versus 6); $P < .7$
5) versus 6); $P < .001$
7) versus 9); $P < .7$
8) versus 9); $P < .001$

TABLE 9. LEUCOCYTE MIGRATION IN THE PRESENCE OF CRUDE HOLOTHURIN AND IODOACETIC ACID

Compound	*Concentration*	*Average migration (in mm)*	*Per cent viable*	*N*
1) Holothurin (1 μg/ml)	8.8×10^{-7}M	1.82	98-100	32
2) Iodoacetic acid	8.0×10^{-5}M	0.98	100	31
3) Holothurin (8.8×10^{-7}M) + IAA	8.0×10^{-5}M	0.91	97-100	31
4) Saline	—	1.66	99-100	31
5) Iodoacetic acid	8.8×10^{-7}M	1.65	100	20
6) Iodoacetic acid	1.0×10^{-4}M	0.26	98-100	20
7) Saline	—	1.53	99-100	18

1) versus 4); $P < .001$
2) versus 3); $P < .3$
2) versus 4); $P < .001$
3) versus 4); $P < .001$
5) versus 7); $P < .2$
6) versus 7); $P < .001$

TABLE 10. LEUCOCYTE MIGRATION IN THE PRESENCE OF CRUDE HOLOTHURIN AND DINITROPHENOL

Compound	*Concentration*	*Average migration (in mm)*	*Per cent viable*	*N*
1) Holothurin	8.8×10^{-7}M	1.90	99-100	35
2) Dinitrophenol	8.0×10^{-5}M	1.65	100	35
3) Holothurin (8.8×10^{-7}M) + DNP	8.0×10^{-5}M	1.78	100	34
4) Saline	—	1.73	100	36
5) Dinitrophenol	1.0×10^{-3}M	1.64	100	26
6) Saline	—	1.60	100	22
7) Dinitrophenol	1.0×10^{-4}M	1.50	95	20
8) Dinitrophenol	8.8×10^{-7}M	1.55	100	15
9) Saline	—	1.50	99-100	25

1) versus 4); $P < .001$
1) versus 3); $P < .02$
2) versus 4); $P < .02$
3) versus 4); $P < .4$
5) versus 6); $P < .6$
8) versus 9); $P < .7$

TABLE 11. LEUCOCYTE MIGRATION IN THE PRESENCE OF CRUDE HOLOTHURIN AND POTASSIUM CYANIDE

Compound	*Concentration*	*Average migration (in mm)*	*Per cent viable*	*N*
1) Holothurin	8.8×10^{-7}M	1.79	98-100	31
2) Potassium cyanide	8.0×10^{-5}M	1.54	99-100	35
3) Holothurin (8.8×10^{-7}M) + KCN	8.0×10^{-5}M	1.50	98	33
4) Saline	—	1.50	99-100	33
5) Potassium cyanide	1.0×10^{-4}M	1.60	99	27
6) Potassium cyanide	1.0×10^{-3}M	1.66	98-99	29
7) Potassium cyanide	1.0×10^{-2}M	0.76	99-100	27
8) Saline	—	1.60	100	25
9) Potassium cyanide	8.8×10^{-7}M	2.14	99-100	30
10) Saline	—	2.14	100	27

1) versus 4); $P < .001$

2) versus 4); $P < .5$

6) versus 8); $P < .7$

7) versus 8); $P < .001$